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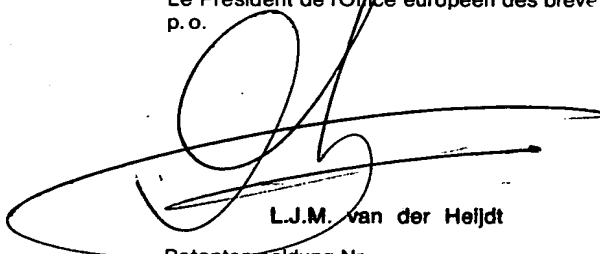
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L.J.M. van der Heljdt

Patentanmeldung Nr. 89202117.1  
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**Blatt 2 der Bescheinigung**  
**Sheet 2 of the certificate**  
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## EFFICIENT PRODUCTION OF MUTANT PROTEASES

### INTRODUCTION

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#### Technical Field

The present invention relates to a method for the production of mutant proteins using recombinant DNA techniques. More specifically, the invention relates to the efficient production of protein engineered Bacillus proteases using a homologous Bacillus host strain, from which the capacity to produce the corresponding unmodified protease has been eliminated.

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#### Background of the Invention

Bacilli are widely used for the production of industrially important enzymes such as  $\alpha$ -amylases, neutral proteases and alkaline (or serine) proteases, see, for example, Debabov: "The industrial use of Bacilli" in: The Molecular Biology of Bacilli, Acad. Press, New York, 1982.

The main advantages of using Bacilli are the large quantities of protein secreted, their lack of production of hazardous substances and their long history of safe industrial use. Several species of Bacilli have therefor been approved for the production of proteins destined for food applications.

The use of Bacillus subtilis as an expression host was demonstrated by Palva et al., Gene 19 (1982) 81-87, describing the expression of the  $\alpha$ -amylase gene from B. amyloliquefaciens in B. subtilis. Other references describing heterologous gene expression in B. subtilis are, e.g., Fahnestock and Fisher, J. Bacteriol. 165 (1986) 796-804, who showed the expression of the Staphylococcus aureus Protein A gene; Schein et al., Biotechnology 4 (1986) 719-725, disclosing the expression of human interferon- $\alpha 2$ ; and Wang et al., Gene 69 (1988) 39-47, showing the expression

and secretion of human atrial natriuretic  $\alpha$ -factor. To ensure the proper secretion of these heterologous proteins the former two references used the  $\alpha$ -amylase signal sequence and the latter reference the signal sequence of the B. subtilis subtilisin gene (aprE) fused to the gene to be expressed.

A major problem of Bacillus strains as production hosts for recombinant proteins is that they produce and secrete a variety of proteases which tend to degrade the heterologous proteins produced. See, e.g., Old and Primrose, "Principles of Gene Manipulation", 3rd ed. (1985) 289, Blackwell, Oxford.

In International Patent Application (PCT) WO 89/04866 the importance of extracellular proteases is pointed out; about 90% of the proteolytic activity is attributed to the neutral metalloprotease (npr) and the serine protease (apr).

Various solutions have been proposed to overcome this degradation problem.

Kawamura and Doi, J. Bacteriol. 160 (1984) 442-444, disclose a B. subtilis double mutant deficient in both extracellular neutral and alkaline protease. First B. subtilis DB100 was made neutral protease negative by transferring the nprR2 and the nprE18 mutations from B. subtilis NT18. A 178 bp fragment containing a part of the apr gene was then deleted by gene conversion.

Fahnestock and Fisher, Appl. Environ. Microbiol. 53 (1987) 379-384, describe the construction of an apr B. subtilis strain starting from a npr strain. They used a plasmid vector containing the apr gene and introduced the cat gene both as an inactivator and a selectable marker.

Sloma *et al.*, J. Bacteriol. 170 (1988) 5557-5563, detected and sequenced a third extracellular protease gene (epr) from B. subtilis. Partial deletion of this gene showed that it contributed only marginally to the extracellular protease activity.

However, none of the referenced B. subtilis strains was completely devoid of extracellular proteolytic activity.

In several publications the production of mutant  
5 proteases is described by Bacillus subtilis host strains which are incapable of expressing the wild-type protease gene, thus avoiding mixtures of mutant and wild-type proteases.

In EP-A-0246678 a B. subtilis strain is disclosed  
10 which is incapable of secreting enzymatically active subtilisin or neutral protease, but which expresses heterologous protease genes isolated or derived from B. amyloliquefaciens. Site-specific saturation mutagenesis was performed on the B. amyloliquefaciens gene and the mutants  
15 were expressed. The negative background of the B. subtilis host strains was obtained by partial deletion of either or both of the original protease genes, or by N-methyl-N'-nitro-N-nitrosoguanidine (NTG) mutagenesis. It was further stressed that the B. subtilis host described is normally  
20 sporulating and that asporogenous mutants are unsatisfactory for the production of (recombinant) proteins.

In WO 86/01825 Bacillus strains are described, in particular B. subtilis, with reduced extracellular protease levels. The alkaline protease gene is inactivated by in  
25 vitro insertion of a gene coding for a protein which confers a phenotypic trait on the microorganism. The plasmid vector containing the inactivated protease gene is transformed to B. subtilis and the inactivated gene replaces the native chromosomal gene by homologous recombination. No expression  
30 and secretion of heterologous proteins is suggested.

WO 88/8033 discloses subtilisin analogs from subtilisin Carlsberg, subtilisin DY, subtilisin BPN', an apr subtilisin from B. subtilis and a subtilisin from B. mesentericus. These mutants are obtained by replacing one or  
35 more amino acids in or near the calcium binding site with negatively charged amino acids (e.g. Asp or Glu). The mutants are expressed in B. subtilis.

WO 89/04866 discloses a B. subtilis strain with low secreted protease activity, which was made by a spoOH mutation in an apr<sup>-</sup>npr<sup>-</sup> double mutant. The obtained triple mutant apr<sup>-</sup>npr<sup>-</sup>spoOH<sup>-</sup> showed very low secreted protease activity.

The references discussed above describe the expression of homologous and heterologous genes in B. subtilis and the construction of protease-negative B. subtilis strains. It has appeared that protease-negative strains made by partial deletion of the protease gene or by insertion of another gene in the protease gene show several drawbacks, e.g.:

- the original gene may be reactivated. If a gene which is homologous to the inactivated chromosomal gene is introduced into the cell (on a plasmid), homologous recombination can lead to reactivation of the gene.

- the original gene, although inactivated, may lead to the production of partial expression products, when the promoter region is still active. This is disadvantageous in terms of metabolic efficiency.

- the introduction of unwanted phenotypical traits, e.g. antibiotic resistance, depending on the inserted gene.

- plasmid instability, due to homology between a genomic sequence and a part of the inserted plasmid.

It is therefore desirable to delete all sequences with possible homology between the chromosome and the plasmid.

There are also other drawbacks for the production of alkaline proteases or other enzymes in B. subtilis. Although Wells et al., Nucleic Acids Res. 11, (1983) 7911-7925 shows that expression of B. amyloliquefaciens subtilisin BPN' in B. subtilis is possible with its own transcription signals, Jacobs et al., Nucleic Acids Res. 13 (1985) 8913-8926 shows that such a strategy is not generally applicable. It was demonstrated that the 5' region of subtilisin Carlsberg derived from B. licheniformis did not contain functional signals for transcription in B. subtilis. To achieve

considerable production it was needed to insert a B. subtilis promoter in front of the gene in question. It is also suggested that not only transcriptional but also translational, secretion or maturation processes may occur less efficient in heterologous hosts, due to a not perfect Shine Dalgarno sequence or even incompatibility of the gene product with the heterologous host.

The problems mentioned above can be avoided when the product is synthesized in a homologous Bacillus strain, of which high production capabilities are proven, with high efficiency resulting in high production levels.

#### SUMMARY OF THE INVENTION

Transformed alkalophilic Bacillus strains are provided for the efficient production strains of mutant Bacillus proteases. The new strains have lost their ability to express their protease encoding genes originally present.

In a preferred embodiment mutant proteases are produced which show close homology with the proteases originally produced by the corresponding strain.

Preferred strains are Bacillus novo species PB92, derivatives thereof and closely related strains, transformed by a mutated protease encoding gene. Said gene shows at least 30%, preferably at least 50% and more preferably at least 80% homology with the wild-type gene of Bacillus PB92 encoding serine protease. The gene is preferably derived from a wild-type gene of an alkalophilic Bacillus strain.

Also provided are methods of constructing the new transformed strains, vectors used thereby and a method of producing mutant proteases.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the construction of plasmid pM58 $\Delta$  containing parts of the 5' and 3' flanking regions of the alkaline protease gene from Bacillus novo sp. PB92.

Figure 2 schematically shows the introduction of the temperature-sensitive origin of replication from plasmid pE194neo-1 into plasmid pM58 $\Delta$ , giving the plasmid pEM58 $\Delta$ .

5 Figure 3 shows the introduction by homologous recombination, of the plasmid pEM58 $\Delta$  into the 5' flanking region of the protease gene of Bacillus strain PBT110.

Figure 4 shows a simplified restriction map of the regions surrounding the deleted protease gene after illegitimate recombination. Two strains were obtained,  
10 PBT125 and PBT126.

Figure 5 nucleotide sequence of the HindIII fragments of PBT125 (A) and PBT126 (B).

Figure 6 schematic representation of the introduction of the protease gene into M13 mp18.

15 Figure 7 shows the nucleotide sequence of plasmid pBHA-1.

Figure 8 shows the introduction of the protease gene of Bacillus strain PBT110 in plasmid pBHA1.

Figure 9 shows the reorientation of the F1 ori containing BamHI fragment of pBHA1-MXL giving plasmid pBHAR-MXL.  
20

Figure 10 shows the subcloning of the 3' sequence of the high alkaline protease into plasmid pBHAR-MXL, giving plasmid pBHARB-MXL.

25 Figure 11 shows the deletion of the E. coli sequence from plasmid pBHARB-MXL M216Q giving plasmid pBHB-MXL M216Q.

Figure 12 shows the reorientation of the neomycin resistance gene in plasmid pE194neo-1 giving plasmid pE194neo-3.

30 Figure 13 schematically shows the introduction of the temperature-sensitive origin of replication from plasmid pE194neo-3 into plasmid pBHB-MXL M216Q giving plasmid pEN<sub>3</sub>Q.

Figure 14 schematically shows the construction of strain Bacillus strain PEP111.

35 Figure 15 schematically shows the construction of strain Bacillus strain PEP211.



# DETAILED DESCRIPTION OF THE INVENTION

For the production of alkaline proteases preferably alkalophilic Bacillus strains are used as host cells. For large scale production industrial strains are a need. They originate from organisms which may be isolated from the soil or are available from depositories or other sources and are obtained by genetic modification of such Bacillus strains. Industrial Bacillus strains are defined in EP-A-134048. They are characterized by being resistant to genetic exchange, such as phage infection or transformation. The strains are stable and may or may not be capable of spore formation. They are usually prototrophic and modified to provide for high yields of endogenous protein products, such as the enzymes alpha-amylase and various proteases. The yield of an endogenous protein product obtained in an industrial production process can amount to at least 5 g/l (0.5% w/v). Industrial strains also secrete DNases, which result in degradation of DNA in the medium, providing for protection against genetic exchange.

An additional advantage of using alkalophilic Bacilli as production strains is the minimal risk of contamination with other microorganisms, because of the alkaline pH during fermentation.

According to the present invention a method is disclosed for the deletion of a Bacillus protease encoding gene from the genome in such a way that reversion becomes impossible. Said deletion may be a partial deletion but more preferably the gene is completely deleted. The protease negative strain obtained by this method is used for the production of homologous mutant proteases after introduction of an expression vector carrying the gene encoding this mutant protease. It is, however, understood that such a protease negative strain will also be advantageous for the expression of other proteins.

It has been found that by using an industrial protease producing Bacillus strain as a host for the

35 High alkaline protease producing bacilli are taxonomically not well classified and are generally referred to as alkalophilic Bacillus strains. For the present

invention we define alkalophilic bacilli as Bacillus strains that grow well under alkaline conditions pH 9-11 (Horikoshi, K. and T. Akiba, 1982, Alkalophilic microorganisms, Springer Verlag, New York). The alkaline proteases produced by such bacilli are also called high alkaline proteases. Examples of Bacillus strains capable of growing at alkaline pH are described in, for example, U.S. Patent Nos. 3,723,250, Re. 30,602 and 4,480,037. An example of an alkalophilic Bacillus host strain is Bacillus novo species PB92 disclosed inter alia in U.S. Patent No. Re. 30,602. Derivatives of these alkalophilic Bacillus strains, that have been optimized for protease production are employed to produce their proteases on industrial scale (See EP-A-0284126). The products are used in several industrial applications e.g. as an additive in laundry detergents. Examples of such products are Maxacal<sup>R</sup> (Gist-brocades/IBIS), Savinase<sup>R</sup> (NOVO), Esperase<sup>R</sup> (NOVO). Mutants of such products have been described in WO 89/06279, where it is said that they are derived from Bacillus lentus strains, and in the not prepublished European Patent Application EP-A-0328229.

According to a preferred embodiment of the present invention transformed Bacillus strains are provided which produce the mutants described in EP-A-0328229 and WO 89/06279, preferably on an industrial scale.

According to the present invention a Bacillus strain is suitably used, in particular an alkalophilic Bacillus, preferably PB92. Another preferred group of Bacillus strains are the asporogenous mutants, of which PBT110 and its derivatives are most preferred. Transformation of alkalophilic Bacillus strains will preferably involve the use of protoplasts from said strains. However, the conventional protoplast transformation protocol as described by Chang, S. and S.M. Cohen, Molec. Gen. Genet. 168 (1979) 111-115, does not work for alkalophilic Bacillus strains. The protocols needed for these strains have been disclosed in EP-A-0283075, which is herein included by reference.

Expression of mutant protease genes in homologous hosts necessitates the replacement and/or inactivation of the wild type gene. Some methods can be used.

A) One method is to clone the gene or part thereof, modify it by site-directed mutagenesis and reintroduce the (partial) gene into the cell on a plasmid. By homologous recombination the gene may be introduced into the chromosome. Resulting in a situation in which a wild-type and the mutant gene are tandemly located. After a second recombination the modified sequence is left in the chromosome having thereby effectively introduced the mutation into the chromosomal gene (as described in Figure 14).

B) In another method the chromosomal gene copy is inactivated by insertion or deletion. If insertional inactivation is chosen as a strategy the DNA fragment used as an insert may be any fragment as long as the formation of a complete protein product from the inactivated gene is prevented. Preferably the fragment contains a selectable marker. If deletion of the gene is chosen as a strategy the chromosomal gene fragment that is deleted can be the whole coding region or a part thereof. After the inactivation a mutant copy of the inactivated gene is brought into the cell on a cloning vector.

C) In another method the chromosomal gene copy is mutagenized. In principle it is possible to obtain (specific) mutations in vivo, after transformation of bacteria with oligonucleotides which are mutagenic. Such a method has been successfully used in Yeast as described by Moerschell et al. Proc. Natl. Acad. Sci. U.S.A. 85 (1988) 524-528. If such a method is performed on a homologous preferably a homologous alkalophilic bacillus strains this is embodiment of this invention.

The present invention relates to Bacillus strains that are known to be efficient protease producers. In one of the preferred methods the complete alkaline protease coding region is deleted from the vector in vitro, leaving behind

35           In yet another embodiment the chromosomal high  
alkaline protease gene is replaced by the mutant gene by



**5**

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL SECTION

EXAMPLE 1

5        Construction of Inactivation Plasmid pEM58  $\Delta$

Plasmid pM58 (see EP-A-0284126) was digested with restriction enzymes BalI and HpaI. The large fragment containing part of the flanking sequences of the protease  
10 gene was purified (Maniatis, Molecular cloning: A Laboratory Manual, Cold Spring Harbor 1982) and ligated. The ligation mix was transformed (Spizizen et al., J. Bacteriol. 81 (1961) 741-746) to Bacillus subtilis DB104 (Kawamura and Doi, J. Bacteriol. 160 (1984) 442-444) and selected for  
15 neomycin resistant and protease negative transformants on minimal plates containing 0,4% casein and 20  $\mu$ g/ml neomycin.

Plasmid pM58  $\Delta$  was isolated and characterized by restriction enzyme analysis. As shown in Figure 1 plasmid pM58  $\Delta$  contains the flanking sequences of the alkaline  
20 protease gene, the gene coding for neomycin resistance and a Bacillus origin of replication.

To construct an integration plasmid containing a temperature sensitive origin of replication, pM58  $\Delta$  was digested with restriction enzymes XbaI and BglII. The  
25 fragment containing the alkaline protease flanking sequences was purified and ligated to the (purified) XbaI/BglII fragment of pE194neo-1 (EP-A-0284126) containing the temperature sensitive origin of replication, derived from pE194 (Jordanescu et al., Plasmid 1 (1978) 468-479) after  
30 said fragment was purified as formerly described. The ligation mix was transformed to B. subtilis DB104 and selected for neomycin resistance (see above). Plasmid pEM58  $\Delta$  was isolated and characterized (Figure 2). It contains the neomycin resistance gene, the temperature sensitive origin  
35 of replication from pE194 and the flanking sequences of the alkaline protease gene.



This plasmid pEM58  $\Delta$  was used to transform Bacillus strain PBT110.

## EXAMPLE 2

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### Construction of a Protease Negative Alkalophilic Bacillus Strain

#### 10 A. Protoplast transformation of Bacillus PBT110 by plasmid pEM58 $\Delta$

Bacillus strain PBT110 is an asporogenous mutant of Bacillus novo species PB92 (U.S. Patent No. Re. 30,602) and was obtained by classical (UV) mutation procedures. This  
15 strain was transformed with plasmid pEM58  $\Delta$  similar to the method described in EP-A-0284126. Prior to integration experiments, transformants were checked by restriction enzyme analysis whether they contained the relevant plasmid.

#### 20 B. Integration of pEM58 $\Delta$ in Bacillus PBT110 chromosome

Integration of pEM58  $\Delta$  in Bacillus strain PBT110 chromosome was performed as described in EP-A-0284126. Selection for integrants occurred at a neomycin concen-  
25 tration of 1  $\mu$ g/ml. The genetic organization of the integrant was determined by restriction enzyme analysis followed by Southern blotting and hybridization analysis and is shown in Figure 3. It appeared that integration of pEM58  $\Delta$  took place through a so-called Campbell-type mechanism by  
30 homologous recombination in the 5' flanking region of the alkaline protease gene, resulting in Bacillus strain PBT110-INT5.

#### 35 C. Selection for protease negative strains

Bacillus strain PBT110-INT5 was inoculated in 100 ml



undefined size appeared to be left in the chromosome after deletion of the protease gene. The sequence of this fragment was determined as follows. The chromosomal HindIII fragments of strains PBT125 and PBT126 containing the plasmid/chromosome junctions were ligated in phage vector M13 mp18 (Messing et al., Nucl. Acids Res. 9 (1981) 303-321) and transfected to E. coli JM101 according to the procedure described by Cohen et al., Proc. Natl. Acad. Sci. USA 69 (1972) 2110-2114. After phage propagation in E. coli JM101, ssDNA was isolated (Heidecker et al., Gene 10 (1980) 69-73).

The inserts were sequenced using the method described by Sanger et al., Proc. Natl. Acad. Sci. USA 74 (1977) 6463. The results are shown in Figures 5A and 5B, respectively.

### EXAMPLE 3

#### Construction of Protease Production and Mutation Vectors

##### A. Subcloning of the PB92 protease gene in phage M13 mp18

Plasmid pM58 was digested with HpaI and BalI. After purification of the DNA fragment containing the protease gene (Maniatis, 1982) this fragment was ligated into phage M13 mp18 which was digested with SmaI. The ligation mixture was transfected to E. coli JM101 (Cohen et al., supra). After phage propagation in E. coli JM101, dsDNA was isolated according to the method described by Birnboim and Doly (Nucl. Acids Res. 7 (1979) 1513-1523). The insert and its orientation were checked by restriction enzyme analysis. Vector mp18-MXL was used for further subcloning experiments and is shown in Figure 6.

##### B. Subcloning of the PB92 protease gene in plasmid pBHA1

Figure 7 shows the nucleotide sequence of plasmid pBHA1. This plasmid is derived from the twin vector system



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pBHA/C-1 was modified to obtain the twin vector system pBHARB-MXL/pBHCRB-MXL, as follows:

1. Vector mp18-MXL was digested with KpnI and HincII. The DNA fragment containing the protease gene was purified  
5 and ligated into pBHA1 which was digested with EcoRV and KpnI. The ligation mixture was transformed to E. coli JM101 (Maniatis, 1982) and plated on LC<sup>+</sup> plates containing 10 g/l trypton, 5 g/l Yeast extract (Difco), 8 g/l NaCl, 25 g/l thymine, 1 g/l MgSO<sub>4</sub>·7H<sub>2</sub>O, 100 µg/ml ampicillin and 20  
10 µg/ml neomycin pH 7.0. Plasmid DNA of transformants was isolated (Birnboim, supra) and characterized by restriction enzyme analysis. In this way pBHA1-MXL was isolated (see Figure 8).

2. In order to sequence mutations introduced in the  
15 protease gene with an available set of oligonucleotides, it was necessary to reverse the orientation of the F1 origin compared to the protease gene. This was performed in the following way: plasmid pBHA1-MXL was digested with BamHI and religated. The ligation mix was transformed to E. coli WK6  
20 (Maniatis, supra) and selected for neomycin or ampicillin resistance on LC<sup>+</sup> plates containing 10 g/l trypton, 5 g/l Yeast Extract (Difco), 8 g/l NaCl, 25 mg/l thymine, 1 g/l MgSO<sub>4</sub>·7H<sub>2</sub>O, 100 µg/ml ampicillin and 20 µg/ml neomycin pH 7.0. Plasmid DNA of transformants was isolated (Birnboim,  
25 supra) and characterized by restriction enzyme analysis. In this way pBHAR-MXL was isolated, which differs from pBHA-MXL in that the orientation of the E. coli sequence is reversed, (see Figure 9).

3. For the introduction of additional flanking  
30 sequences in plasmid pBHAR-MXL, the following digestions were made. Plasmid pM58 was digested with SphI and HindIII. Plasmid pBHAR-MXL (Figure 10) was digested with SphI and HindIII. The larger fragment was purified. Both digests were ligated and transformed to B. subtilis DB104 and selected  
35 for neomycin resistance and protease activity on minimal plates containing 10 µg/ml neomycin and 0.4 % casein. Transformants were characterized by restriction enzyme

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analysis. One of these, pBHARB-MXL (Figure 10), was used for further experiments.

C. Mutagenesis of the PB92 gene in plasmid pBHARB-MXL

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Mutagenesis was carried out with the twin vector system pBHARB-MXL/pBHCRB-MXL (Stanssens, supra).

A method based on the gapped-duplex approach (Kramer  
et al., Nucl. Acids Res. 12 (1984) 9441) and a phasmid  
10 (phage/plasmid hybrid) was used. Essentially the method  
rests on a gapped-duplex DNA intermediate consisting of a  
gapped strand (- strand) containing a wild-type antibiotic  
resistance marker and a template strand (+ strand) carrying  
an amber mutation in the gene conferring resistance to the  
15 antibiotic. After annealing, the mutagenic oligonucleotide  
becomes incorporated in the gapped strand during in vitro  
gap-filling and sealing reaction. The resultant molecules  
are used to transform a mismatch repair deficient (Mut S)  
host in which the linkage between the intended mutation and  
20 the antibiotic resistance marker is preserved. The mixed  
phasmid population, isolated from this strain, is then  
allowed to segregate in a suppressor negative host strain.  
Transformants are plated on antibiotic containing medium,  
thus imposing a selection for progeny derived from the  
25 gapped strand.

In the pMa type vector nucleotide 3409 is changed  
from G to A, while in the pMc type vector nucleotide 2238 is  
changed from G to C, creating amber stopcodons in the  
chloramphenicol-acetyltransferase gene and  $\beta$ -lactamase  
30 gene, respectively, rendering said genes inactive.

To perform mutagenesis the target DNA fragment is  
cloned into the multiple cloning site of pMa5-8 or a  
derivative thereof. A gapped duplex between pMa5-8  
35 containing the target DNA and pMc5-8 is then constructed.

The single strand gap, consisting of the target DNA,  
may be subjected to mutagenesis with a mutagenic oligo-



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described in EP-A-0284126. Prior to production studies, transformants were checked by restriction enzyme analysis to contain the relevant plasmid.

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# EXAMPLE 5

## Construction of Protease Integration Vectors

### A. Integration vector pEN<sub>3</sub>Q

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Plasmid pE194neo-1 (EP-A-0284126) was digested with SalI and religated. The ligate was transformed to B. subtilis DB104 and selected for neomycin resistance on minimal plates containing 20 µg/ml neomycin. Plasmid pE194  
15 neo-3 was isolated and checked on the presence of the neomycin resistance gene in a reversed orientation (see Figure 12). Plasmid pE194neo-3 was digested with HpaI and BglII. The fragment containing the temperature sensitive origin of replication was purified (Maniatis, supra).

20

Plasmid pBHB-MXL M216Q (see Example 3 and Figure 11) was digested with BglII and BalI. The fragment containing the protease gene was purified. Both fragments were ligated. The ligation mix was transformed to B. subtilis DB104 and selected for protease activity and neomycin resistance as  
25 described before. Transformants were characterized by restriction enzyme digestions. One of these, containing plasmid pEN<sub>3</sub>Q, was selected (Figure 13). This plasmid contained the neomycin resistance gene, the t.s. origin of replication from plasmid pE194neo-3 and the M216Q mutation  
30 in the PB92 protease gene.

### B. Integration vector pEN<sub>3</sub>S.

In the same way as described for integration vector  
35 pEN<sub>3</sub>Q, pEN<sub>3</sub>S was constructed using pBHARB-MXL M216S as starting vector.



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## EXAMPLE 6

### Construction of Bacillus Strains PEP111 and PEP112

5    A.    Protoplast transformation of Bacillus PBT110 by Plasmid pEN<sub>3</sub>Q

Bacillus strain PBT110 was transformed with plasmid pEN<sub>3</sub>Q as described in EP-A-0284126. Prior to integration  
10    experiments, it was checked by restriction enzyme analysis whether the transformants contained the relevant plasmid.

          B.    Integration of pEN<sub>3</sub>Q in Bacillus PBT110 chromosome

15            Integration experiments with plasmid pEN<sub>3</sub>Q in Bacillus strain PBT110 chromosome were performed as described in EP-A-0284126. Selection for integrants occurred at a neomycin concentration of 1µg/ml at non-permissive temperatures (50°C) for plasmid replication. To  
20    check the integration of pEN<sub>3</sub>Q in the chromosome, chromosomal DNA of potential integrants was isolated, digested with HindIII or ClaI, run on a 0.8% DNA agarose gel and blotted to nitrocellulose (Southern, J. Mol. Biol. 98 (1975) 503-517) and hybridized with <sup>32</sup>P labeled nick-  
25    translated pEN<sub>3</sub>Q DNA (Maniatis, 1982).

          It was shown that integration of pEN<sub>3</sub>Q had occurred by homologous recombination resulting in a strain (PBT110M/Q) with two protease genes (one wild type and one mutant M216Q) tandemly located on the chromosome. Thus,  
30    integration of pEN<sub>3</sub>Q occurred by a so-called Campbell-type mechanism as depicted in Figure 14 (see also EP-A-0284126).

          C.    Selection of neomycin sensitive recombinants

35            Neomycin sensitive recombinants were selected, which contain either the wild-type or the mutant protease gene in the chromosome (hereinafter called "outrecombinants"). The

Strain PBT110 M/Q was used as starting strain. After strain PBT 110 M/Q was incubated in PBT minimal medium, the culture was plated on PBT minimal medium plates containing 0.4% casein and 15 g/l agar. These plates were incubated for 48 h at 37°C and replica plated to Heart infusion (HI) plates containing 1 µg/ml neomycin and no neomycin, respectively. Neomycin sensitive colonies were considered outrecombinants.

Chromosomal DNA of potential outrecombinants was isolated, digested with ClaI or HindIII, run on a 0.5% agarose gel, blotted to nitrocellulose (Southern, 1975) and hybridized with <sup>32</sup>P labeled nick translated pEN<sub>3</sub>Q (Maniatis, 1982). Since mutation M216Q results in the removal of a ClaI site, the strains containing a wild-type protease or a mutant protease gene, and the intermediate strain PBT110 M/Q can be easily distinguished, see Fig. 14.

An outrecombinant containing the M216Q mutant protease gene of PB92 was isolated. After it was shown that a gene replacement of PB92 wild-type protease to PB92 mutant protease had occurred, the product of the strain was characterized by its biochemical parameters ( $k_{cat}$ ,  $K_m$ ), oxidation resistance, specific activity and wash performance, as described EP-A-0328229. All results were equal to a control protease M216Q, indicating that the product of the transformed strain was indeed the PB92 protease containing the mutation M216Q. This transformed strain is called Bacillus PEP111.

Similar experiments were performed using vector pEN<sub>3</sub>S to construct Bacillus strain PEP112, which contains  
35 the PB92 protease with mutation M216S.

#### EXAMPLE 7

##### Construction of Bacillus Strains PEP211 and PEP212

5        Bacillus strain PEP111 was transformed with plasmid  
pEN<sub>3</sub>Q, following the procedure described in EP-A-0284126.  
Prior to the integration procedure, it was checked by  
restriction enzyme analysis whether the transformants  
10 and the selection for integrants (at a neomycin  
concentration of 20 µg/ml at non-permissive temperatures  
(50°C) for plasmid replication) was performed as described  
in EP-A-0284126.

15        To check the integration of plasmid pEN<sub>3</sub>Q in the  
chromosome, chromosomal DNA of potential integrants was  
isolated and digested with HindIII, run on 0.8% agarose  
gels, blotted to nitrocellulose (Southern et al. 1979) and  
hybridized with <sup>32</sup>P labeled nick translated pEN<sub>3</sub>Q. It was  
20 shown that a strain containing two mutant (M216Q) PB<sub>92</sub>  
protease genes, separately located on the chromosome was  
isolated. This strain is called PEP211. Its chromosomal  
organization is shown in Figure 15.

25        To obtain an analogous strain containing two protease  
genes with mutation M216S, strain PEP112 containing a mutant  
gene (M216S) after gene replacement of the wild-type gene,  
was transformed with plasmid pEN<sub>3</sub>S and procedures were  
followed as described above. These experiments resulted in  
strain PEP212 containing two separately located PB<sub>92</sub> mutant  
M216S protease genes on the chromosome.

30

#### EXAMPLE 8

##### Production of Mutant Proteases

35        Production of protease mutants was performed with  
transformed Bacillus strains in which the mutated protease

encoding gene was either localized on a plasmid or on the chromosome.

For plasmid encoded production 2 types of strains were used, the alkalophilic strain Bacillus PBT125, as  
5 herein described, and B. subtilis strain DS12367, an asporogenic strain derived from B. subtilis DB104 (Kawamura et al., J. Bacteriol. (1984) 160, 442-444. These strains were transformed with pBHB-MXL derived plasmids. Bacillus PBT125 and B. subtilis DS12367, which do not possess  
10 protease encoding plasmids, were used as control strains.

For production with strains containing chromosomally integrated protease mutant genes the Bacillus PEP strains, as herein described, were used. Bacillus PBT strains 108 and 110, containing the wild-type protease genes were used as  
15 control strains. PBT108 is a strain containing two wild-type genes separately located on the chromosome, see EP-A-0284126. For PBT110, see Example 2A.

To check the activity of the original promoter of the PB92 protease gene in B. subtilis, a construct was made,  
20 pBHB-MXLR, in which the orientation of the protease gene to the HpaII promoter (Zyprian et al., DNA 5 (1986) 219-225) was reversed, so that the expression of the protease gene is only directed by its original promoter.

The productions were performed in 500 ml shake flasks  
25 containing 100 ml protease-production medium.

When alkalophilic Bacillus strains were used cultures were incubated, after inoculation, for 40 hours at 37°C in a production medium as described in U.S. Patent No. Re. 30,602. In case of plasmid containing strains, neomycin (20  
30 µg/ml) was added.

When B. subtilis DS12367 strains were used, a comparable medium containing 12.5 g/l Yeast Extract (Difco), 0.97 g/l CaCl<sub>2</sub>·6H<sub>2</sub>O, 2.25 g/l MgCl<sub>2</sub>·6H<sub>2</sub>O, 20 mg/l MnSO<sub>4</sub>·4H<sub>2</sub>O, 1 mg/l CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.5 g/l citrate, 0.5 ml antifoam 5693, 6%  
35 w/w maltose, 0.2 M phosphate buffer pH 6.8 was applied. In case of plasmid containing strains, neomycin (20 µg/ml) was added.

Cultures containing B. subtilis strains were incubated for 65 hours at 37°C. In all cases the shake-flasks were inoculated with 0.1 ml of a Tryptic Soya Broth culture containing 20 µg/ml neomycin containing the relevant strain which had been incubated for 24 hours at 37°C.

Protease activity was assayed using dimethylcasein as substrate as described by Lin et al., J. Biol. Chem. 244 (1969) 789-793. The specific activities of the protease mutants (EP-A-0328229) were used to determine the production on mg basis.

The results of the fermentation experiments are summarized in the following Table 1.

Table 1

15

	<u>Strain</u>	<u>Relative protease production (%)</u>	<u>Mutation</u>
20	PBT110	100	WT
	PBT125	0	--
	PBT108	120	WT
	PEP111	100	M216Q
25	PEP112	100	M216S
	PBT125 pBHB-MXL	95-100	WT
	PBT125 pBHB-MXL M216Q	95-100	M216Q
	PBT125 pBHB-MXL M216S	95-100	M216S
	PBT125 pBHB-MXL S160D	95-100	S160D
30	PBT125 pBHB-MXL N212D	95-100	N212D
	PEP211	120	M216Q
	PEP212	120	M216S
	DS12367	0-1	--
35	DS12367 pBHB-MXL	4	WT
	DS12367 pBHB-MXL	40	WT
	DS12367 pBHB-MXL M216Q	40	M216Q
	DS12367 pBHB-MXL M216S	40	M216S
	DS12367 pBHB-MXL S160D	40	S160D
40	DS12367 pBHB-MXL N212D	40	N212D

All publications (including patents and patent applications) mentioned in this specification are indicative to the level of skill of those skilled in the art to which this invention pertains. All publications are

herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

5 Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

## CLAIMS

1. A method for the production of proteases differing in at least one amino acid from the wild-type protease, said method comprising the use of a homologous alkalophilic Bacillus strain as expression host said strain being unable to produce the wild-type protease.

2. A method according to Claim 1, employing as  
10 expression host a protease negative alkalophilic Bacillus  
strain.

3. A method according to Claim 2, employing as expression host a protease negative alkalophilic Bacillus strain, wherein the wild-type protease gene is deleted from the genome.

4. A method according to any one of Claims 1 to 3,  
employing a protease negative derivative of Bacillus novo  
20 species PB92.

5. A method according to any one of Claims 1 to 4, employing an asporogenic alkalophilic Bacillus strain.

25           6. A method according to any one of Claims 1 to 5,  
employing an asporogenic Bacillus from which the wild-type  
protease gene has been deleted by homologous or illegitimate  
recombination.

30           7. A method according to any one of Claims 2 to 6,  
wherein the mutant high alkaline protease is plasmid  
encoded.

8. A method according to Claim 1, 2 or 3, wherein the  
35 wild-type high alkaline protease has essentially the amino  
acid sequence of Bacillus novo species PB92 protease.

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9. A method according to Claim 1, wherein the wild-type alkaline protease gene is deleted from the chromosome, and at least one copy of a mutant high alkaline protease gene is inserted into the genome.

5

10. A method according to Claim 9, wherein at least one copy of a mutant high alkaline protease gene is inserted into the genome and another copy is plasmid encoded.

10

11. A method of obtaining an alkalophilic Bacillus strain having a reduced extracellular alkaline protease level comprising:

15

- isolating a gene, including the 5' and/or 3' noncoding regions thereof, coding for alkaline protease,
- inserting the gene into a cloning vector,
- deleting the coding region of the gene from the cloning vector,
- transforming a Bacillus strain with the cloning vector,
- growing the transformants under conditions in which the replication function of the vector is inactive,
- selecting transformants which have lost the DNA sequence encoding the alkaline protease.

20

12. A method according to Claim 11, wherein said alkalophilic Bacillus strain is Bacillus novo species PB92 or a derivative thereof.

25

13. An alkalophilic Bacillus strain obtained according to Claim 11, transformed with a plasmid expression vector containing a mutant high alkaline protease gene.

14. A Bacillus strain according to Claim 13, wherein the alkalophilic Bacillus strain is a derivative of Bacillus novo species PB92, obtained by homologous or illegitimate recombination.

30

35



15. An alkaline protease differing in at least one amino acid from the wild-type protease produced by a Bacillus strain having at least one copy of the mutated gene and no copy of the wild-type gene giving an active protease.

5

16. An alkaline protease according to Claim 15, produced by an alkalophilic Bacillus strain.

17. An alkaline protease according to Claim 15 or 16,  
10 where the mutant gene is provided on a plasmid and/or in the genome.

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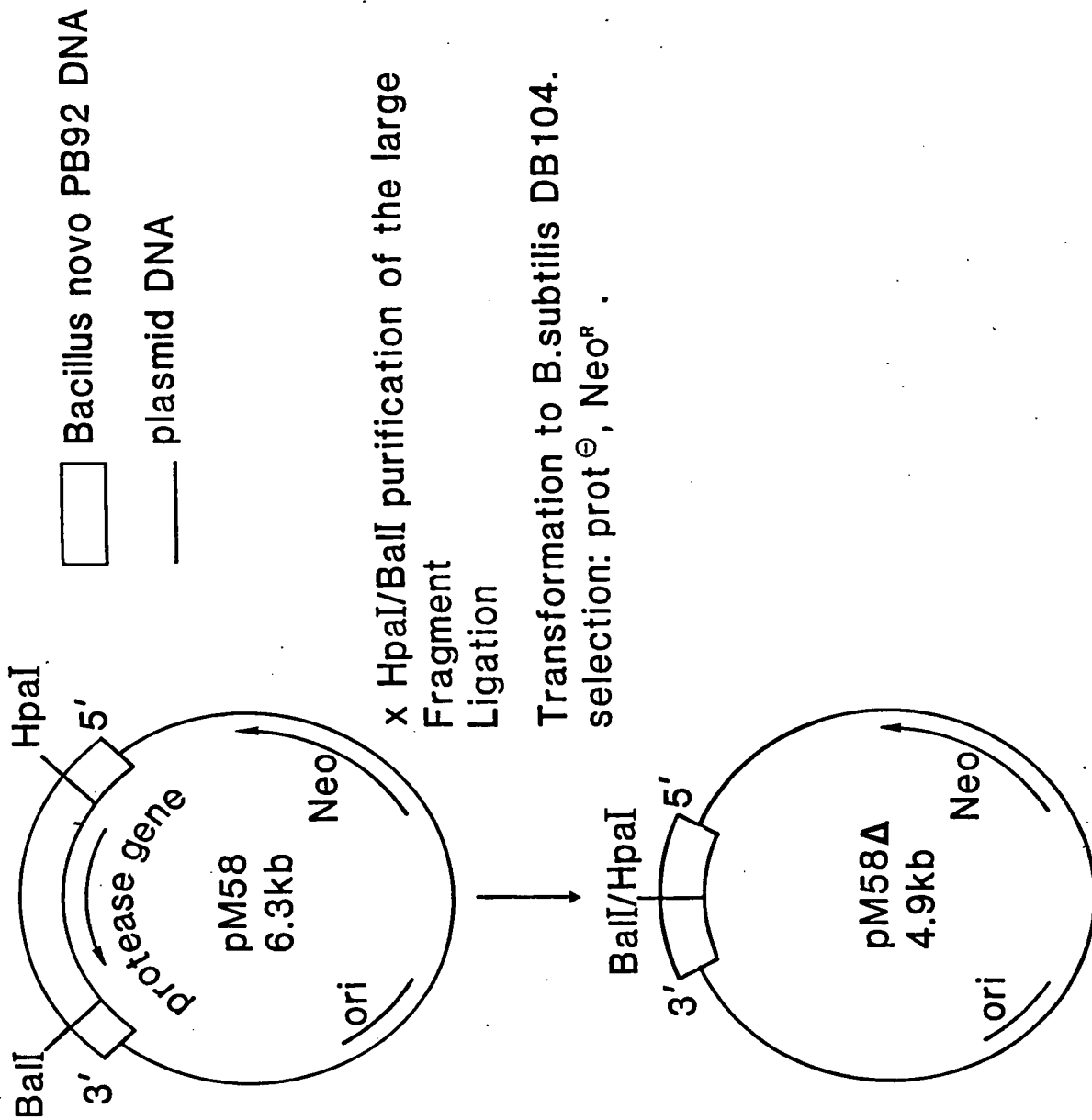
EFFICIENT PRODUCTION OF MUTANT PROTEASES

5

Abstract of the Disclosure

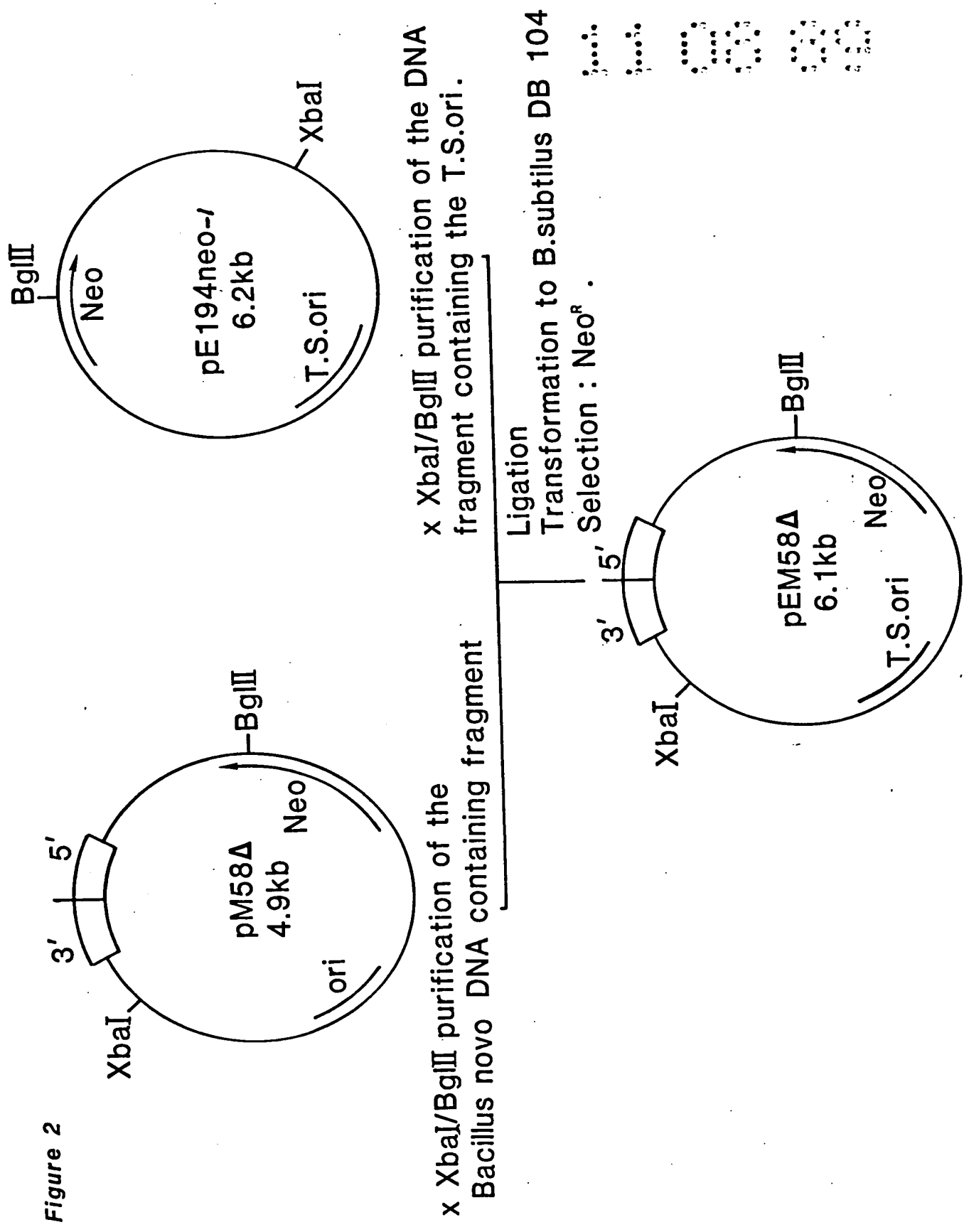
Alkalophilic Bacillus strains are provided which efficiently produce mutant Bacillus proteases and which are incapable of expressing the wild-type proteases.

Figur 1



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Figure 2







HindIII . . . . . 40 . . . . . 80  
AAGCTTTTCAAATGATCCAGGAGAACAAAAACGATTTTTTTGAGGAAAGTTATAAATTATTTTCCGAAACGATATGGCAAGC  
 . . . . . 120 . . . . . 160  
 AAAATATTGCTTATGCAACAGTTCATAATGATGAGCAAACCCCTCACATGCATTTAGGTGTTGTGCCTATGCGTGATGGA  
 . . . . . 200 . . . . . 240  
 AAACTGCAAGGAAAAAATGTGTTTAATCGTCAAGAACTGTTATGGCTACAAGATAAATTCCCCGAGCATATGAAAAACA  
 . . . . . 280 . . . . . 320  
 GGGTTTTGAGTTGAAGCGTGGTGAACGTGGCTCTGACCGTAAACATATTGAGACAGCTAAATTTAAAAAACAACCTTTGG  
 . . . . . XbaI . . . . . 360 . . . . . 400  
 AAAAAGAGATTGATTTTCTAGAAAATGAATACGGTAAGGAAAATATGCTGTATGCGACTGTCCATCTGGATGAAAGAGTC  
 . . . . . 440 . . . . . 480  
 CCACATATGCACCTTTGGTTTTGTCCCTTTAACAGAGGACGGGAGATTGTCTGCAAAGAACAGTTAGGCCAACAGAAAGA  
 . . . . . 520 . . . . . 560  
 CTTTACTCAATTACAAGATAGATTTAATGAGTATGTGAATGAGAAAGGTTATGAACTTGAAAGAGGCACGTCCAAAGAGG  
 . . . . . 600 . . . . . 640  
 TTACAGAACGAGAACATAAAGCGATGGATCAGTACAAGAAAGATACTGTATTTTCATAAACAGGAACTGCAAGAAGTTAAG  
 . . . . . 680 . . . . . 720  
 GATGAGTTACAGAAGGCAAATAAGCAGTTACAGAGTGGAATAGAGCATATGAGGTCTACGAAACCCTTTGATTATGAAAA  
 . . . . . 760 . . . . . 800  
 TGAGCGTACAGGTTTGTCTCTGGACGTGAAGAGACTGGTAGAAAGATATTAAGTCTGATGAATTTGAACGCCCTGCAAG  
 . . . . . PstI . . . . . 840 . . . . . 880  
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 . . . . . 920 . . . . . 960  
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 . . . . . 1000 . . . . . 1040  
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 . . . . . 1240 . . . . . 1280  
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 . . . . . 1320 . . . . . 1360  
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 . . . . . 1400 . . . . . 1440  
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 . . . . . 1560 . . . . . 1600  
 AATTTCTAGGCAACAACAACGCCGCTATTCTTGACGGTGGGATTTCAGGCTTGAAGAACAGTGGCTACCCGACAACGGGA  
 . . . . . HindIII  
 TGAGGTGAAGCTT

figure 5A

HindIII . . . . . 40 . . . . . 80  
AAGCTTTTCAAATGATCCAGGAGAACAAAAACGATTTTTTTGAGGAAAGTTATAAATTATTTTCCGAACGATATGGGAAGC  
 . . . . . 120 . . . . . 160  
 AAAATATTGCTTATGCAACAGTTCATAATGATGAGCAAACCCCTCACATGCATTTAGGTGTTGTGCCTATGCGTGATGGA  
 . . . . . 200 . . . . . 240  
 AAAGTCAAGGAAAAAATGTGTTTAATCGTCAAGAACTGTTATGGCTACAAGATAAATCCCCGAGCATATGAAAAACA  
 . . . . . 280 . . . . . 320  
 GGGTTTTGAGTTGAAGCGTGGTGAACGTGGCTCTGACCGTAAACATATTGAGACAGCTAAATTTAAAAAACAACCTTTGG  
 . . . . . XbaI . . . . . 360 . . . . . 400  
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 . . . . . 440 . . . . . 480  
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 . . . . . 520 . . . . . 560  
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 . . . . . 600 . . . . . 640  
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 . . . . . 680 . . . . . 720  
 GATGAGTTACAGAAGGCAAATAAGCAGTTACAGAGTGGAATAGAGCATATGAGGTCTACGAAACCCTTTGATTATGAAAA  
 . . . . . 760 . . . . . 800  
 TGAGCGTACAGGTTTGTCTCTGACGTGAAGAGACTGGTAGAAAGATATTAAGTCTGATGAATTTGAACGCCTGCAAG  
 . . . . . PstI . . . . . 840 . . . . . 880  
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 . . . . . 1320 . . . . . 1360  
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 . . . . . 1560 . . . . . 1600  
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 . . . . . 1640 . . . . . 1680  
 AGAAAATAGCGATTATGAACGTCGTTGGCGGTACATTTACCGACGAGCCATATGGGGATTGCTGTCCGCAAACGGCGACG  
 . . . . . EcoRI . . . . . 1720 . . . . . HindIII  
ACGAATTCACGCGAAAAAATCAATGAAATCTTGCGAGACTTAAAGCTT

figure 5B



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Figure 6

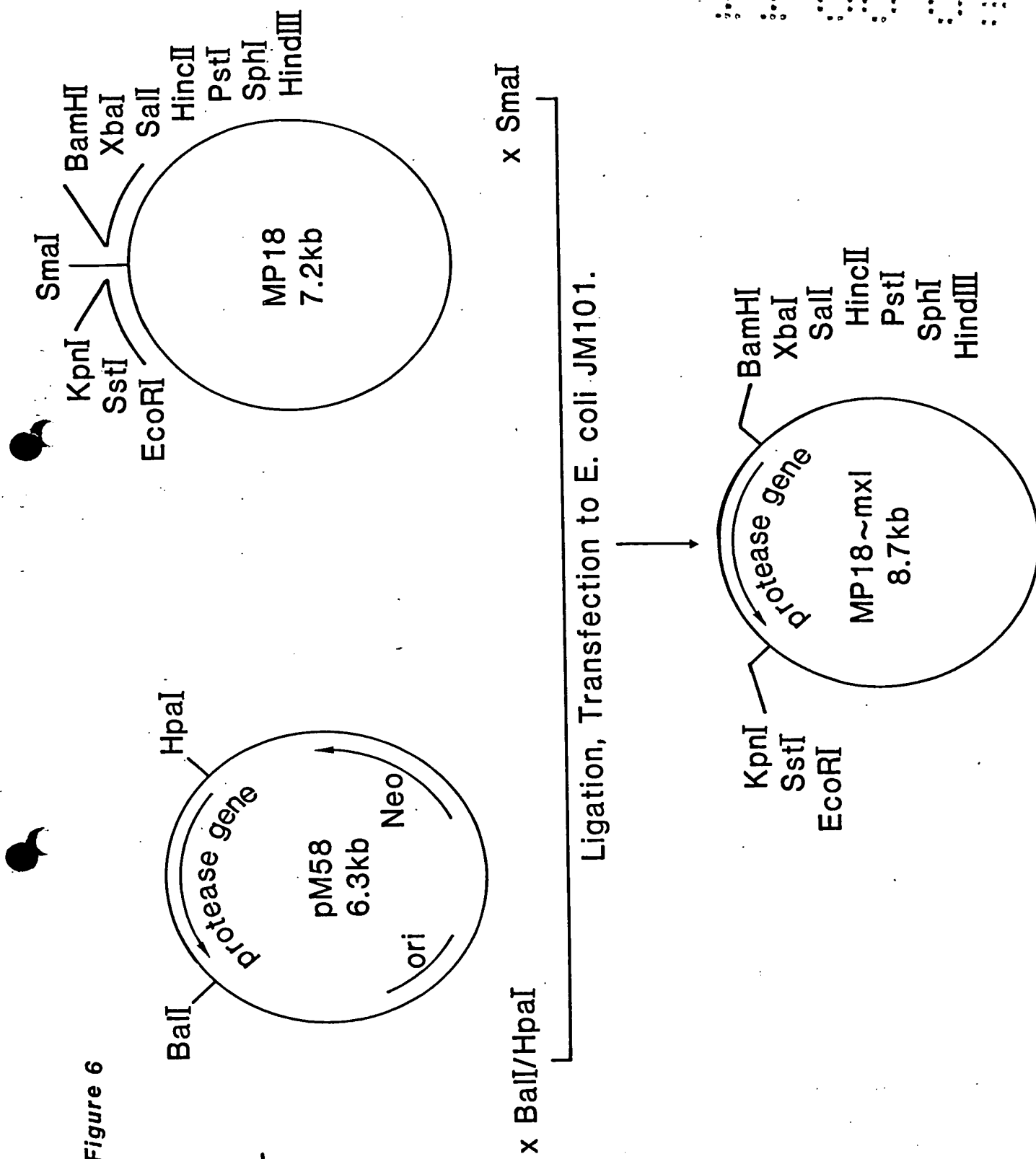


figure 7

Sequence of pBHA-1

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30
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60
TTTTTTGGAG ATTTTCAACG TGAAAAAATT ATTATTCGCA ATTCCAAGCT AATTCACCTC
90
GAAAGCAAGC TGATAAACCG ATACAATTAA AGGCTCCTTT TGGAGCCTTT TTTTTTGGAG
120
ATTTTCAACG TGAAAAAATT ATTATTCGCA ATTCCAAGCT CTGCCTCGCG CGTTTCGGTG
150
ATGACGGTGA AAACCTCTGA CACATGCAGC TCCCGGAGAC GGTCACAGCT TGTCTGTAAG
180
CGGATGCAGA TCACGCGCCC TGTAGCGGCG CATTAAAGCGC GGCGGGTGTG GTGGTTACGC
210
GCAGCGTGAC CGCTACACTT GCCAGCGCCC TAGCGCCCGC TCCTTTCGCT TTCTTCCCTT
240
CCTTTCTCGC CACGTTTCGCC GGCTTTCCCC GTCAAGCTCT AAATCGGGGG CTCCCTTTAG
270
GGTTCCGATT TAGTGCTTTA CGGCACCTCG ACCCCAAAAA ACTTGATTAG GGTGATGGTT
300
CACGTAGTGG GCCATCGCCC TGATAGACGG TTTTTCGCCC TTTGACGTTG GAGTCCACGT
330
TCTTTAATAG TGGACTCTTG TTCCAAACTG GAACAACACT CAACCCTATC TCGGTCTATT
360
CTTTTGATTT ATAAGGGATT TTGCCGATTT CGGCCTATTG GTTAAAAAAT GAGCTGATTT
390
AACAAAAATT TAACGCGAAT TTAAACAAAA TATTAACGTT TACAATTGTA TCTGCGCTCG
420
GTCGTTTCGGC TGCGGCGAGC GGTATCAGCT CACTCAAAGG CGGTAATACG GTTATCCACA
450
GAATCAGGGG ATAACGCAGG AAAGAACATG TGAGCAAAAG GCCAGCAAAA GGCCAGGAAC
480
CGTAAAAAGG CCGCGTTGCT GGCGTTTTTC CATAGGCTCC GCCCCCCTGA CGAGCATCAC
510
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540
TTTCCCCCTG GAAGCTCCCT CGTGCGCTCT CCTGTTCCGA CCCTGCCGCT TACCGGATAC
570
600
630
660
690
720
750
780
810
840
870
900
930
960
990
1020
1050
1080

```



110300

2250  
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2370 2400  
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2430 2460  
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2550 2580  
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2670 2700  
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2730 2760  
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2790 2820  
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2850 2880  
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2910 2940  
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2970 3000  
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3030 3060  
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3090 3120  
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3150 3180  
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3210 3240  
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3270 3300  
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3330 3360  
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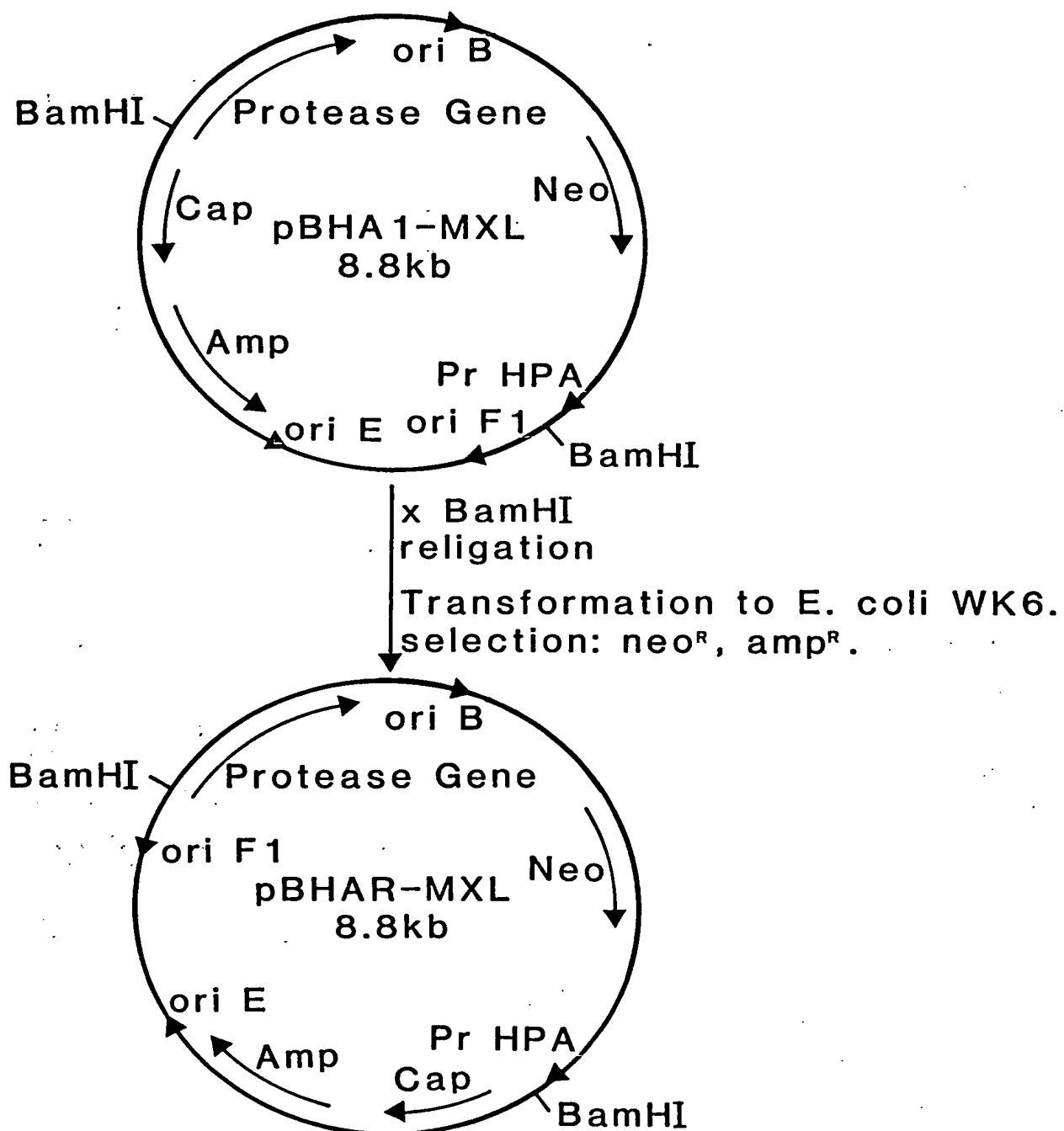






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Figure 9



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Figure 10

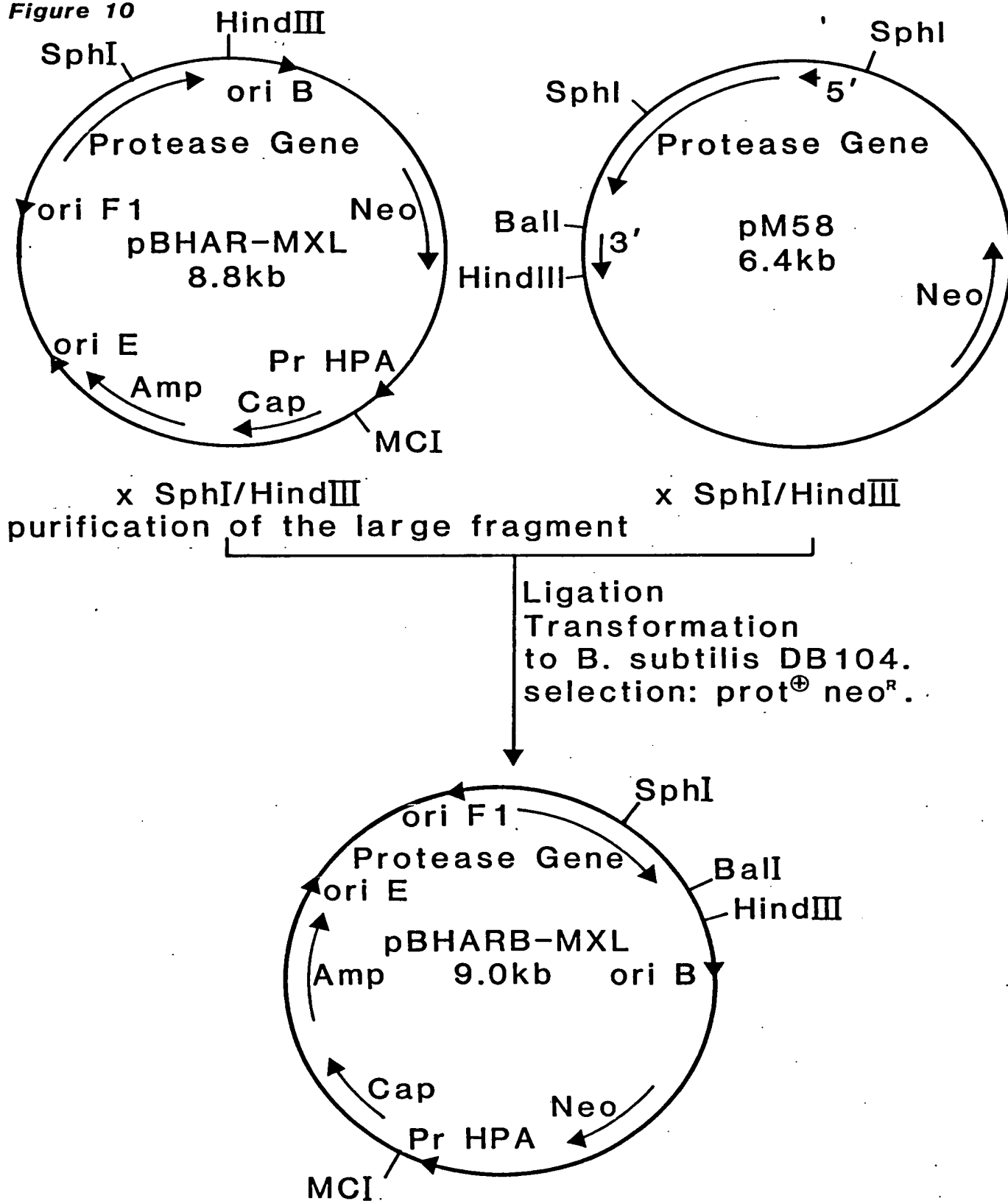
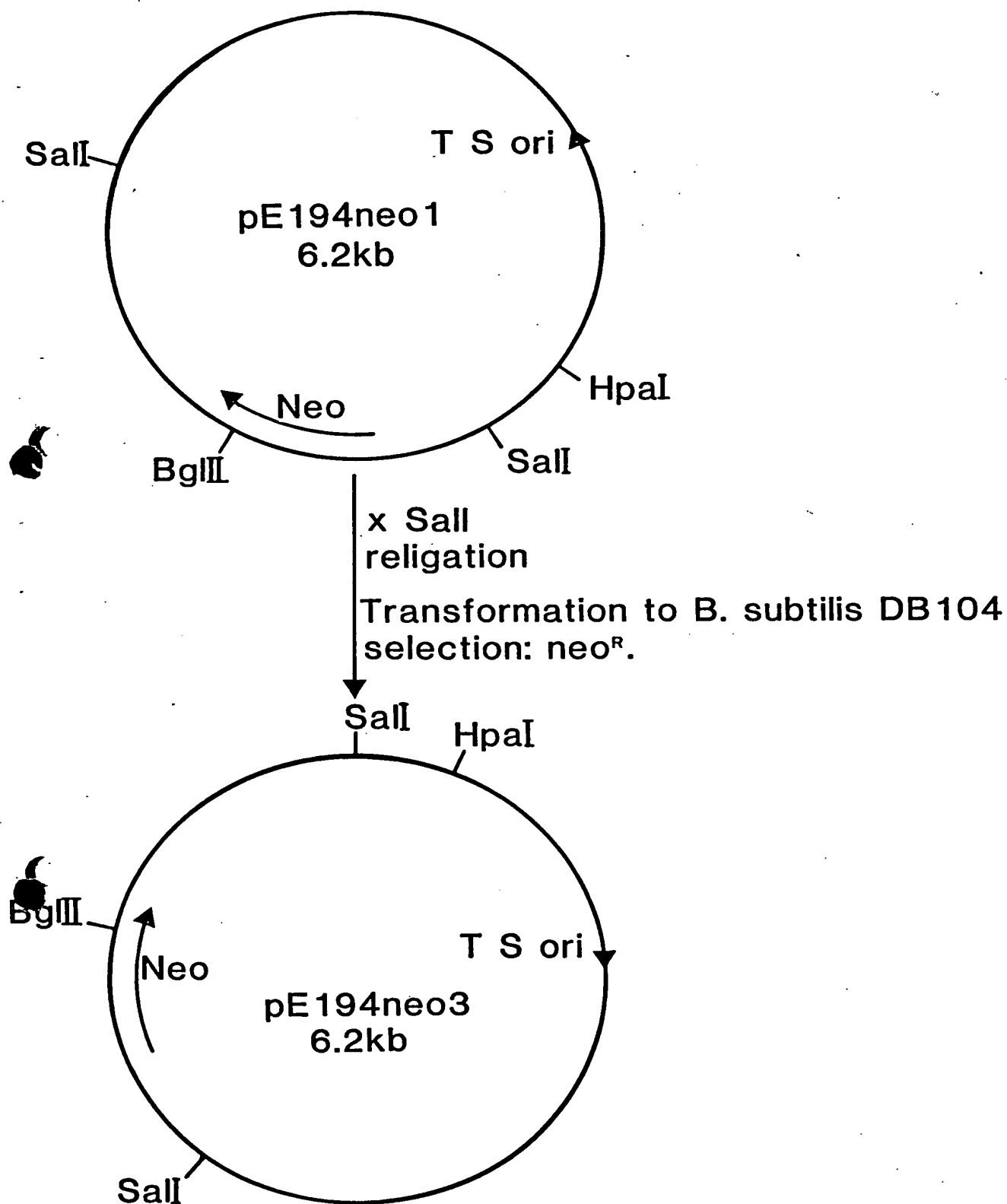




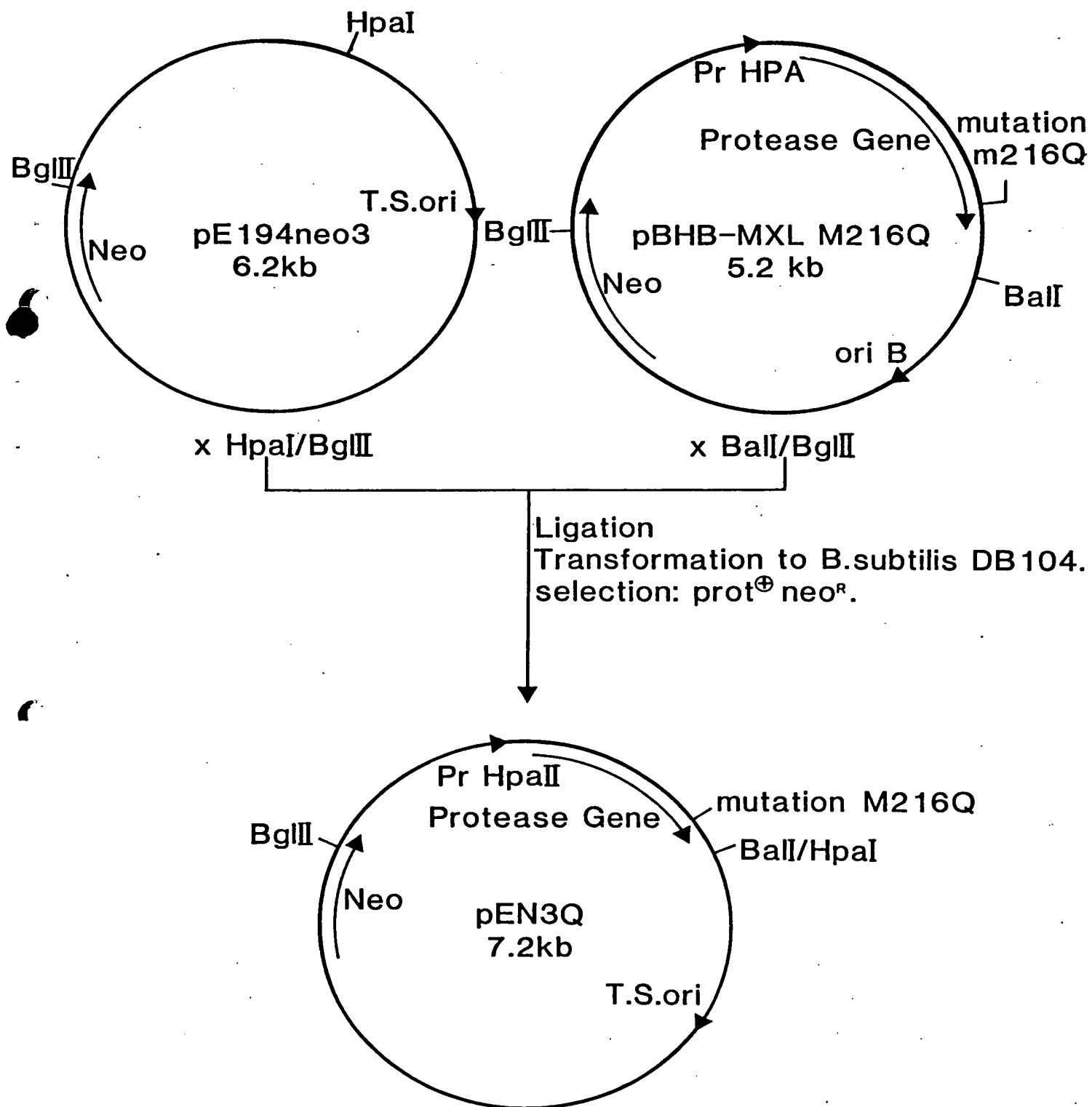
Figure 12

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Figure 13



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Figure 14

